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(54) Title: PLASMIDS CONTAINING DNA-SEQUENCES THAT CAUSE CHANGES IN THE CARBOHYDRATE CONCENTRATION AND THE CARBOHYDRATE COMPOSITION IN PLANTS, AS WELL AS PLANT CELLS AND PLANTS CONTAINING THESE PLASMIDS

(57) Abstract

Plasmids are described having DNA sequences that after insertion into the genome of the plants cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants. These changes can be obtained from a sequence of a branching enzyme that is located on these plasmids. This branching enzyme alters the amylose/amylopectin ratio in starch of the plants, especially in commercially used plants.

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Plasmids containing DNA-Sequences that cause changes in the carbohydrate concentration and the carbohydrate composition in plants, as well as plant cells and plants containing these plasmids

The present invention relates to plasmids containing DNA-sequences which contain information that, after insertion into a plant genome, cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, as well as plant cells and plants containing sequences from these plasmids.

Because of the continual growth in word population, there is a continually growing demand for nutrient and raw materials. It is the task of biotechnological research to achieve a change of the content as well as yield of crops. To do this the metabolism of the plants has to be altered.

A particular interest is the possibility of using plant ingredients as renewable raw material sources e.g. for the chemical industry. This is especially of great importance for two reasons. Firstly, up to now, mineral oil and coal deposits have been the main source of raw materials for the petrochemical industry but these deposits are finite and it can be seen that alternative, renewable raw material sources must be developed.

Secondly, the present situation of agriculture in Europe and North America has lead to a surplus of crops grown for their nutritive properties. This causes obvious financial and political problems in agriculture. Alternative products for which there is a higher quantitative demand could be a solution to this problem.
Renewable raw materials can be divided into fats and oils, proteins and carbohydrates, such as mono-, di-, oligo- and

polysaccharides. The most important polysaccharides are starch and cellulose. In the EEC, the total starch production in 1987-1988 comprised maize (60%), wheat (19%) and potato (21%).

For an increasing use of plant starch as an industrial raw material the quality of the starch must meet the demands of the processing industry. Important considerations include the amylose to amylopectin ratio, the chain length, the branching grade of the amylopectin as well as the size of the starch granules.

The main biochemical synthetic pathways for the production of starch in higher plants are well known. Starch consists of amylose and amylopectin, in which the amylose consists of a linear α-1,4-glucan and amylopectin consists of α-1,4-glucans, which are connected to each other via α 1,6-linkages and thus form a branched polyglucan. The so-called branching enzyme (Q-enzyme) is responsible for the introduction of the α-1,6-linkage. One method for the production of starch which only has a linear α-1,4-glucan structure is therefore by the inhibition of the enzymatic activity of the proteins and/or the inhibition of the biosynthesis of the branching enzyme. New biotechnology processes for the genetic alteration of dicotyledonous and monocotyledonous plants by transfer and stable installation of single isolated genes or groups of genes are known (Gasser and Fraley, Science 244, 1293-1299). The possibility of specific expression of foreign genes inserted in the plant by gene technology, primarily in potato tubers, is also known (EP 375092 and Rocha-Sosa et al., EMBO J. 8, 23-29 (1989)).

The present invention provides plasmids containing DNA-sequences which contain information that, after

insertion into a plant genome, cause changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants.

The invention further provides plant cells containing sequences from these plasmids which can be regenerated to whole plants, as well as plants containing sequences from these plasmids.

The term "plant" means a commercially useful plant, preferably maize, barley, wheat, rice, peas, soya beans, sugar cane, sugar beet, tomato, potato or tobacco.

Carbohydrates which can be altered by the DNA sequences are mono-, di-, oligo- or polysaccharides. Starch is an example of a polysaccharide which can be modified in plants and plant cells.

With the plasmids of the invention, it is possible to modify the amylose to amylopectin ratio of the starch in plant cells and in plants. This is possible through the presence of a branching enzyme, located on the plasmid, which has the following sequence:

```
          10        20        30        40        50        60
   1  TCAGGAGCGGTCTTGGGATATTTCTTCCACCCCAAAATCAAGAGTTAGAAAAGATGAAAG
  61  GATGAAGCACAGTTCAGCTATTTCCGCTGTTTTGACCGATGACAATTCGACAATGGCACC
 121  CCTAGAGGAAGATGTCAACACTGAAAATATTGGCCTCCTAAATTTGGATCCAACTTTGGA
 181  ACCTTATCTAGATCACTTCAGACACAGAATGAAGAGATATGTGGATCAGAAAATGCTCAT
 241  TGAAAAATATGAGGGACCCCTTGAGGAATTTGCTCAAGGTTATTTAAAATTTGGATTCAA
 301  CAGGGAAGATGGTTGCATAGTCTATCGTGAATGGGCTCCTGCTGCTCAGGAAGCAGAAGT
 361  TATTGGCGATTTCAATGGTAGGAACGGTTCTAACCACATGATGGAGAAGGACCAGTTTGG
 421  TGTTTGGAGTATTAGAATTCCTGATGTTGACAGTAAGCCAGTCATTCCACACAACTCCAG
 481  AGTTAAGTTTCGTTTCAAACATGGTAATGGAGTGTGGGTAGATCGTATCCCTGCTTGGAT
 541  AAAGTATGCCACTGCAGACGCCACAAAGTTTGCAGCACCATATGATGGTGTCTACTGGGA
 601  CCCACCACCTTCAGAAAGGTACCACTTCAAATACCCTCGCCCTCCCAAACCCCGAGCCCC
 661  ACGAATCTACGAAGCACATGTCGGCATGAGCAGCTCTGAGCCACGTGTAAATTCGTATCG
 721  TGAGTTTGCAGATGATGTTTTACCTCGGATTAAGGCAAATAACTATAATACTGTCCAGTT
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781 GATGGCCATAATGGAACATTCTTACTATGGATCATTTGGATATCATGTTACAAACTTTTT
841 TGCTGTGAGCAATAGATATGGAAACCCGGAGGACCTAAAGTATCTGATAGATAAAGCACA
901 TAGCTTGGGTTTACAGGTTCTGGTGGATGTAGTTCACAGTCATGCAAGCAATAATGTCAC
961 TGATGGCCTCAATGGCTTTGATATTGGCCAAGGTTCTCAAGAATCCTACTTTCATGCTGG
1021 AGAGCGAGGGTACCATAAGTTGTGGGATAGCAGGCTGTTCAACTATGCCAATTGGGAGGT.
1081 TCTTCGTTTCCTTCTTTCCAACTTGAGGTGGTGGCTAGAAGAGTATAACTTTGACGGATT
1141 TCGATTTGATGGAATAACTTCTATGCTGTATGTTCATCATGGAATCAATATGGGATTTAC
1201 AGGAAACTATAATGAGTATTTCAGCGAGGCTACAGATGTTGATGCTGTGGTCTATTTAAT
1261 GTTGGCCAATAATCTGATTCACAAGATTTTCCCAGACGCAACTGTTATTGCCGAAGATGT
1321 TTCTGGTATGCCGGGCCTTAGCCGGCCTGTTTCTGAGGGAGGAATTGGTTTTGATTACCG
1381 CCTGGCAATGGCAATCCCAGATAAGTGGATAGATTATTTAAAGAATAAGAATGATGAAGA
1441 TTGGTCCATGAAGGAAGTAACATCGAGTTTGACAAATAGGAGATATACAGAGAAGTGTAT
1501 AGCATATGCGGAGAGCCATGATCAGTCTATTGTCGGTGACAAGACCATTGCATTTCTCCT
1561 AATGAACAAAGAGATGTATTCTGGCATGTCTTGCTTGACAGATGCTTCTCCTGTTGTTGA
1621 TGCAGGAATTGCGCTTGACAAGATGATCCATTTTTTTCACAATGGCCTTGGGAGGAGAGG
1681 GGTACCTCAATTTCATGGGTAACGAGTTTGGCCATCCTGAGTGGATTGACTTCCCTAGTG
1741 AGGGCAATAATTGGAGTTATGACAAATGTAGACGCCAGTGGAACCTCGCAGATAGCGAAC
1801 ACTTGAGATACAAGTTTATGAATGCATTTGATAGAGCTATGAATTCGCTCGATGAAAAGT
1861 TCTCATTCCTCGCATCAGGAAAACAGATAGTAAGCAGCATGGATGATGATAATAAGGTTG
1921 TTGTGTTTGAACGTGGTGACCTGGTATTTGTATTCAACTTCCACCCAAATAACACATACG
1981 AAGGGTATAAAGTTGGATGTGACTTGCCAGGGAAGTACAGAGTTGCACTGGACAGTGATG
2041 CTTGGGAATTTGGTGGCCATGGAAGAGCTGGTCATGATGTTGACCATTTCACATCACCAG
2101 AAGGAATACCTGGAGTTCCAGAAACAAATTTCAATGGTCGTCCAAATTCCTTCAAAGTGC
2161 TGTCTCCTGCGCGAACATGTGTGGCTTATTACAGAGTTGATGAACGCATGTCATAAACTG
2221 AAGATTACCAGACAGACATTTGTAGTGAGCTACTACCAACAGCCAATATCGAGGAAAGTG
2281 ACGAGAAACTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAATGCTT
2341 ATTACAGAGTTGATGAACGCATGTCAGAAGCTGAAGATTACCAGACAGACATTTGTAGTG
2401 AGCTACTACTACCAACAGCCAATATCGAGGAGAGTGACGAGAAACTTGATGATTCATTAT
2461 CTACAAATATCAGTAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAAGG
2521 AACTTAAAGATTCACCATCTGTAAGCATCATTAGTGATGCTGTTCCAGCTGAATGGGCTG
2581 ATTCGGATGCAAACGTCTGGGGTGAGGACTAGTCAGATGATTGATCGATCCTTCTACGTT
2641 GGTGATCTCGGTCCGTGCATGATGTCTTCAGGGTGGTAGCATTGACTGATTGCATCATAG
2701 TTTTTTTTTTTTTTTTTAAGTATTTCCTCTATGCATATTATTAGCATCCAATAAATTTAC
2761 TGGTTGTTGTACATAGAAAAAGTGCATTTGCATGTATGTGTTTCTCTGAAATTTTCCCCA
2821 GTTTTGGTGCTTTGCCTTTGGAGCCAAGTCTCTATATGTAATAAGAAAACTAAGAACAAT
2881 CACATATATAAAATGTTAGTAGATTACCA .

The property of the branching enzyme to modify the amylose/amylopectin ratio in starch is not limited to a coding sequence exactly as it is shown here but can also be represented by slightly different nucleotid sequences. The property of the branching enzyme is also not changed when the plasmids containing the branching enzyme, are modified in the plant cell or the plant.

To be active, the DNA sequence of the branching enzyme is fused to the regulatory sequences of other genes which guarantee a transcription of the DNA (coding) sequence of the branching enzyme. The DNA sequence can also be fused in an inverted direction to the regulatory sequences of other genes, whereby the 3'-end of the coding sequence is fused to the 3'-end of the promoter and the 5'-end of the coding sequence is fused to the 5'-end of the termination signal. In this way an anti-sense RNA of the branching enzyme is produced in the plant. The regulatory sequences are hereby promoters and termination signals of plant or viral genes, such as for example the promoter of the 35S RNA of the cauliflower mosaic virus or the promoter of the class I patatin-gene B 33 and the termination signal of the 3'-end of the octopine synthase gene of the T-DNA of the Ti-plasmid pTiACH5.

Plant cells containing sequences from these plasmids can be regenerated in known manner to complete transgenic plants. It is possible to insert simultaneously, more than one copy of these sequences into a plant cell or plant.

The following plasmids were deposited at the Deutsche Sammlung von Mikroorganismen (DSM) in Braunschweig, Germany on the 20th August 1990 (deposit number):

Plasmid	P35 S-BE	(DSM 6143)
Plasmid	P35 S-anti-BE	(DSM 6144)
Plasmid	P33-BE	(DSM 6145)
Plasmid	P33-anti-BE	(DSM 6146)

Description of the Figures

Figure 1 shows the restriction map of the 13.6 kb plasmid P35 S-BE. The plasmid contains the following fragments.

A = Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909-7437 of the cauliflower mosaic virus.

B = Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.

C = Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 from the nucleotide 11749 to 11939.

Also shown are the cleavage sites described in Example 1.

Figure 2 shows the restriction map of the 13.6 kb plasmid P35 S-anti-BE. The plasmid contains the following fragments:

A = Fragment A (529 bp) contains the 35S promoter of the cauliflower mosaic virus (CaMV). The

fragment contains the nucleotides 6909 to 7437 of the CaMV.

B = Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.

C = Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotides 11749-11939.

Also shown are the cleavage sites described in Example 2.

Figure 3 shows the restriction map of the 14.6 kb plasmid P33-BE. The plasmid contains the following fragments.

A = Fragment A (1526 bp) contains the DraI-DraI-fragment of the promoter region of the patatin-gene B33. The fragment contains the nucleotide positions -1512 to +14.

B = Fragment B (2909 bp) contains the DNA fragment which codes for the branching enzyme.

C = Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotide positions 11749-11939.

Also shown are the cleavage sites described in Example 3.

Figure 4 shows the restriction map of the 14.6 plasmid P33-anti-BE. Plasmid contains the following fragments:

A = Fragment A (1526 bp) contains the DraI-DraI

fragment of the promoter region of the patatin gene B 33. The fragment contains the nucleotide position -1512 to +14.

B = Fragment B (2909 bp) contains the cDNA-fragment which codes for the branching enzyme.

C = Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5. The fragment contains the nucleotides 11749-11939.

Also shown are the cleavage sites described in Example 4.

In order to understand the examples forming the basis of this invention all the processes necessary for these tests and which are known per se will first of all be listed:

1. Cloning process

The vectors pUC18/19 and pUC118, and the M13mp10 series (Yanisch-Perron et al., Gene (1985), 33, 103-119) were used for cloning.

For plant transformation, the gene constructions were cloned into the binary vector BIN19 (Bevan, Nucl. Acids Res. (1984), 12, 8711-8720).

2. Bacterial strains

The E. coli strain BMH71-18 (Messing et al., Proc. Natl. Acad. Sci. USA (1977), 24, 6342-6346) or TB1 was used for the pUC and M13 mP vectors.

For the vector BIN19 exclusively the E. coli strain TB1 was used. TB1 is a recombinant-negative, tetracycline-resistant derivative of strain JM101

(Yanisch-Perron et al., Gene (1985), 33, 103-119). The genotype of the TB1 strain is (Bart Barrel, personal communication): F'(traD36, proAB, lacI, lacZΔM15), Δ(lac, pro), SupE, thiS, recA, Sr1::Tn10(TcR).

The transformation of the plasmids into the potato plants was carried out by means of the Agrobacterium tumefaciens strain LBA4404 (Bevan, M., Nucl. Acids Res. 12, 8711-8721, (1984); BIN19 derivative).

3. Transformation of Agrobacterium tumefaciens

In the case of BIN19 derivatives, the insertion of the DNA into the agrobacteria was effected by direct transformation in accordance with the method developed by Holsters et al., (Mol. Gen. Genet. (1978), 163, 181-187). The plasmid DNA of transformed agrobacteria was isolated in accordance with the method developed by Birnboim and Doly (Nucl. Acids Res. (1979), 7, 1513-1523) and was separated by gel electrophoresis after suitable restriction cleavage.

4. Plant transformation

10 small leaves, wounded with a scalpel, of a sterile potato culture were placed in 10 ml of MS medium with 2 % sucrose containing from 30 to 50 µl of an Agrobacterium tumefaciens overnight culture grown under selection. After from 3 to 5 minutes gentle shaking, the Petri dishes were incubated in the dark at 25°C. After 2 days, the leaves were laid out on MS medium with 1.6 % glucose, 2 mg/l of zeatin ribose, 0.02 mg/l of naphthylacetic acid, 0.02 mg/l of gibberellic acid, 500 mg/l of claforan, 50 mg/l of kanamycin and 0.8 % Bacto agar. After incubation for one week at 25°C and 3000 lux, the claforan

concentration in the medium was reduced by half. The regeneration and cultivation of the plants were carried out according to known processes (Rocha-Sosa et al EMBO Journal 8, 23-29 (1989).

5. Analysis of genomic DNA from transgenic potato plants

The isolation of genomic plant DNA was effected in accordance with Rogers and Bendich (Plant Mol. Biol. (1985), 5, 69-76.

For the DNA analysis, after suitable restriction cleavage, 10 to 20 µg of DNA were analysed by means of Southern blots for the integration of the DNA sequences to be investigated.

6. Analysis of the total RNA from transgenic potato plants

The isolation of plant total RNA was carried out in accordance with Logemann et al. (Analytical Biochem. (1987), 163, 16-20).

For the analysis, 50 µg portions of total RNA were investigated by means of Northern blots for the presence of the transcripts sought.

7. Protein extraction

For the extraction of total protein from plant tissue, pieces of tissue were homogenised in protein extraction buffer (25 mM sodium phosphate pH 7.0, 2 mM sodium hydrogen sulphite), with the addition of 0.1 % (w/v) of insoluble polyvinylpyrrolidone (PVP).

After filtration through cellulose, cell detritus was centrifuged off for 20 minutes at 10,000 revolutions per minute and the protein concentration of the

supernatant was determined in accordance with the method developed by Bradford (Anal. Biochem. (1976)/ 72, 248-254).

8. Detection of foreign proteins by means of immunological processes (Western blot)

The protein extracts were separated according to molecular weight by means of gel electrophoresis in SDS-PAGE (sodium dodecylsulphate polyacrylamide) gels. After SDS-PAGE the protein gels were equilibrated for from 15 to 30 minutes in transfer buffer for graphite electrodes (48 g/l of tris, 39 g/l of glycine, 0.0375 % SDS, 20 % methanol) and then transferred in a cooling chamber to a nitrocellulose filter and separated at 1.3 mA/cm^2 for from 1 to 2 hours. The filter was saturated for 30 minutes with 3 % gelatin in TBS buffer (20 mM tris/HCl pH 7.5, 500 mM NaCl), and the filter was then incubated for 2 hours with the appropriate antiserum in a suitable dilution (1:1000 - 10000 in TBS buffer) at room temperature. The filter was then washed for 15 minutes each with TBS, TTBS (TBS buffer with 0.1% polyoxyethylene-(20)-sorbitan monolaurate) and TBS buffer. After being washed, the filter was incubated for 1 hour at room temperature with alkaline phosphatase-conjugated goat-anti-rabbit (GAR) antibodies (1:7500 in TBS). The filter was then washed as described above and equilibrated in AP buffer (100 mM tris/HCl pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$). The alkaline phosphatase reaction was started by means of the substrate addition of 70 µl of 4-nitrotetrazolium (NBT) solution (50 mg/ml of NBT in 70 % dimethyl-formamide) and 35 µl of 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (50 mg/ml BCIP in dimethylformamide) in 50 ml of AP buffer. As a rule

the first signals were observed after 5 minutes.

9. Determination of the amylose/amylopectin ratio in starch of transgenic potato plants.

Leaf pieces, having a diameter of 10 mm were floated in 6% sucrose solution under continuous light for 14 hours. This light incubation induced a strong increased starch formation in the leaf pieces. After incubation, the amylose and amylopectin concentration was determined according to Hovenkamp-Hermelink et al (Potato Research 31, 241-246 (1988).

The following examples illustrate the preparation of the plasmids according to the invention, the insertion of sequences from those plasmids into the plant cell as well regeneration of transgenic plants and the analysis of those transgenic plants.

Example 1

Preparation of the plasmid P35s-Be and insertion of the plasmid into the plant genome of the potato.

From a cDNA library in the expression vector λgt11, different clones were identified that cross-react with an antibody that is directed against the branching enzyme of potatoes. These clones were used to identify complete clones from a cDNA library in the HindII-position the vector pUC 19 that originate from isolated mRNA of growing potato tubers. One clone isolated in this manner had an insert size of 2909 bp of the sequence:

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          10         20         30         40         50         60
   1 TCAGGAGCGGTCTTGGGATATTTCTTCCACCCCAAAATCAAGAGTTAGAAAAGATGAAAG
  61 GATGAAGCACAGTTCAGCTATTTCCGCTGTTTTGACCGATGACAATTCGACAATGGCACC
 121 CCTAGAGGAAGATGTCAACACTGAAAATATTGGCCTCCTAAATTTGGATCCAACTTTGGA
 181 ACCTTATCTAGATCACTTCAGACACAGAATGAAGAGATATGTGGATCAGAAAATGCTCAT
 241 TGAAAAATATGAGGGACCCCTTGAGGAATTTGCTCAAGGTTATTTAAAATTTGGATTCAA
 301 CAGGGAAGATGGTTGCATAGTCTATCGTGAATGGGCTCCTGCTGCTCAGGAAGCAGAAGT
 361 TATTGGCGATTTCAATGGTAGGAACGGTTCTAACCACATGATGGAGAAGGACCAGTTTGG
 421 TGTTTGGAGTATTAGAATTCCTGATGTTGACAGTAAGCCAGTCATTCCACACAACTCCAG
 481 AGTTAAGTTTCGTTTCAAACATGGTAATGGAGTGTGGGTAGATCGTATCCCTGCTTGGAT
 541 AAAGTATGCCACTGCAGACGCCACAAAGTTTGCAGCACCATATGATGGTGTCTACTGGGA
 601 CCCACCACCTTCAGAAAGGTACCACTTCAAATACCCTCGCCCTCCCAAACCCCGAGCCCC
 661 ACGAATCTACGAAGCACATGTCGGCATGAGCAGCTCTGAGCCACGTGTAAATTCGTATCG
 721 TGAGTTTGCAGATGATGTTTTACCTCGGATTAAGGCAAATAACTATAATACTGTCCAGTT
 781 GATGGCCATAATGGAACATTCTTACTATGGATCATTTGGATATCATGTTACAAACTTTTT
 841 TGCTGTGAGCAATAGATATGGAAACCCGGAGGACCTAAAGTATCTGATAGATAAAGCACA
 901 TAGCTTGGGTTTACAGGTTCTGGTGGATGTAGTTCACAGTCATGCAAGCAATAATGTCAC
 961 TGATGGCCTCAATGGCTTTGATATTGGCCAAGGTTCTCAAGAATCCTACTTTCATGCTGG
1021 AGAGCGAGGGTACCATAAGTTGTGGGATAGCAGGCTGTTCAACTATGCCAATTGGGAGGT
1081 TCTTCGTTTCCTTCTTTCCAACTTGAGGTGGTGGCTAGAAGAGTATAACTTTGACGGATT
1141 TCGATTTGATGGAATAACTTCTATGCTGTATGTTCATCATGGAATCAATATGGGATTTAC
1201 AGGAAACTATAATGAGTATTTCAGCGAGGCTACAGATGTTGATGCTGTGGTCTATTTAAT
1261 GTTGGCCAATAATCTGATTCACAAGATTTTCCCAGACGCAACTGTTATTGCCGAAGATGT
1321 TTCTGGTATGCCGGGCCTTAGCCGGCCTGTTTCTGAGGGAGGAATTGGTTTTGATTACCG
1381 CCTGGCAATGGCAATCCCAGATAAGTGGATAGATTATTTAAAGAATAAGAATGATGAAGA
1441 TTGGTCCATGAAGGAAGTAACATCGAGTTTGACAAATAGGAGATATACAGAGAAGTGTAT
1501 AGCATATGCGGAGAGCCATGATCAGTCTATTGTCGGTGACAAGACCATTGCATTTCTCCT
1561 AATGAACAAAGAGATGTATTCTGGCATGTCTTGCTTGACAGATGCTTCTCCTGTTGTTGA
1621 TGCAGGAATTGCGCTTGACAAGATGATCCATTTTTTTCACAATGGCCTTGGGAGGAGAGG
1681 GGTACCTCAATTTCATGGGTAACGAGTTTGGCCATCCTGAGTGGATTGACTTCCCTAGTG
1741 AGGGCAATAATTGGAGTTATGACAAATGTAGACGCCAGTGGAACCTCGCAGATAGCGAAC
1801 ACTTGAGATACAAGTTTATGAATGCATTTGATAGAGCTATGAATTCGCTCGATGAAAAGT
1861 TCTCATTCCTCGCATCAGGAAAACAGATAGTAAGCAGCATGGATGATGATAATAAGGTTG
1921 TTGTGTTTGAACGTGGTGACCTGGTATTTGTATTCAACTTCCACCCAAATAACACATACG
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                10        20        30        40        50        60
1981  AAGGGTATAAAGTTGGATGTGACTTGCCAGGGAAGTACAGAGTTGCACTGGACAGTGATG
2041  CTTGGGAATTTGGTGGCCATGGAAGAGCTGGTCATGATGTTGACCATTTCACATCACCAG
2101  AAGGAATACCTGGAGTTCCAGAAACAAATTTCAATGGTCGTCCAAATTCCTTCAAAGTGC
2161  TGTCTCCTGCGCGAACATGTGTGGCTTATTACAGAGTTGATGAACGCATGTCATAAACTG
2221  AAGATTACCAGACAGACATTTGTAGTGAGCTACTACCAACAGCCAATATCGAGGAAAGTG
2281  ACGAGAAACTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAATGCTT
2341  ATTACAGAGTTGATGAACGCATGTCAGAAGCTGAAGATTACCAGACAGACATTTGTAGTG
2401  AGCTACTACTACCAACAGCCAATATCGAGGAGAGTGACGAGAAACTTGATGATTCATTAT
2461  CTACAAATATCAGTAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAAGG
2521  AACTTAAAGATTCACCATCTGTAAGCATCATTAGTGATGCTGTTCCAGCTGAATGGGCTG
2581  ATTCGGATGCAAACGTCTGGGGTGAGGACTAGTCAGATGATTGATCGATCCTTCTACGTT
2641  GGTGATCTCGGTCCGTGCATGATGTCTTCAGGGTGGTAGCATTGACTGATTGCATCATAG
2701  TTTTTTTTTTTTTTTTTAAGTATTTCCTCTATGCATATTATTAGCATCCAATAAATTTAC
2761  TGGTTGTTGTACATAGAAAAAGTGCATTTGCATGTATGTGTTTCTCTGAAATTTTCCCCA
2821  GTTTTGGTGCTTTGCCTTTGGAGCCAAGTCTCTATATGTAATAAGAAAACTAAGAACAAT
2881  CACATATATAAAATGTTAGTAGATTACCA .
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The 2909 bp long c-DNA contained in this clone was used for the next examples and is called cBE.

For the preparation of a plasmid p35s-BE, this cDNA was provided with the promoter of the 35s-RNA of the cauliflower mosaic virus as well as the polyadenylation signal of the octopine synthase gene of the Ti-plasmid pTiACH5. For this the orientation of the C-DNA coding for the branching enzyme was chosen in such a way that the coding strain will be readable (sense-orientation). The plasmid p35s-BE has a size of 13.6 kb and comprises the three fragments A, B and C which were cloned into the cleavage sites of the polylinker of BIN19.

Fragment A (529 bp) contains the 35s promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucleotides 6909 to 7437 of the CaMV (Franck et al., Cell 21, 285-294). It was isolated as EcoRI-KpnI-fragment from the plasmid pDH51 (Pietrzak et al, Nucleic Acids Research 14, 5857-5868) and was cloned between the EcoRI-KpnI-cleavage position of the polylinker of the plasmid BIN 19.

Fragment B contains a 2909 bp cDNA fragment cBe which codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment of the vector pUC 19 and was cloned into the SmaI-position of the polylinker of BIN 19 after filling-in of the Hind-III-position with DNA polymerase. For this the orientation of the cDNA was chosen in such a way that the coding strand is readable and a sense-RNA is formed. The cleavage sites BamHI/XbaI and PstI/SphI originate from the polylinker of pUC 19. The cleavage sites BamHI/XbaI/ SalI/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sites located on the fragment B are internal cleavage sites of the fragment.

Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J. 3, 835,846), nucleotides 11749-11939, which are isolated as PvuII-HindIII fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209-213) and were then cloned onto the PvuII cleavage site between the SphI-Hind-III cleavage site of the polylinker of BIN 19, after addition of SphI linkers (see Fig 1).

The plasmid p35s-BE was transferred into potatoes with the help of the agrobacterial system. After this whole plants were regenerated. Protein extracts isolated from tubers of these plants were tested for the existence of the branching enzyme using the western blot analysis. Further, tubers of these plants were tested for the content of amylose and amylopectin.

Example 2
Preparation of the plasmid p35s-anti-BE and introduction of the plasmid the plant genome of potato.

In a similar manner to that described in Example 1, the plasmid p35s-anti-BE was prepared, but the orientation of the designated cDNA of the branching enzyme was inverted relative to the 35 S promotor. The plasmid p35s-anti-BE has a size of 13.6 kb and comprises the three fragments A, B and C which were cloned in the cleavage sites of the polylinker of BIN19.

Fragment A (529 bp) contains the 35s promoter of the cauliflower mosaic virus (CaMV). The fragment contains the nucl otides 6909 to 7437 of the CaMV (Franck et al. Cell 21, 285-294), and was isolated as EcoRI-KpnI-fragment from the plasmid pDH51 (Pietrzak et al Nucleic Acids Research 14, 5857-5868) and cloned between the EcoRI-KpnI-cleavage

site of the polylinker of the plasmid BIN 19.

Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut from the HindIII-SmaI-fragment of the vector pUC 19 and cloned in the SmaI-position of the polylinker BIN 19 after filling in of the HindIII-position with DNA polymerase. The orientation was chosen in such a way that the non-coding strand is readable and an anti-sense-RNA is formed. The cleavage sites SphI, PstI and XbaI, BamHI, SmaI originate from the polylinker pUC 19. The cutting positions BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sides contained on the fragment B are internal cleavage sides of this fragment.

Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the TI-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846), nucleotides 11749-11939, which were isolated as PvuII-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983), and which were cloned between the SphI-HindIII-cleavage position of the polylinker of BIN 19 after addition of Sph-I-linkers to the Pvu-II-cleavage position (see Fig 2).

The plasmid p35s-anti-BE was transferred into potatoes using the agrobacterial system. After this whole plants were regenerated.

Protein extracts, which had been isolated from tubers of these plants, were tested for the existence of the branching enzyme using the western blot analysis. Tubers of these plants were also tested for the content of amylose and amylopectin.

Example 3
Preparation of the plasmid p33-BE and introduction of the plasmid into the plant genome of the potato.

In a similar manner to that described in Example 1, the plasmid p33-BE was prepared, but replacing the 35s promoter with the promoter of the class I patatin-gene B33 (Rocha-Sosa et al EMBO J 8 23-29). The plasmid p33-Be has a size of 14.6 kb and consists of the three fragments A, B and C that were cloned into the cleavage position of the polylinker of BIN 19.

Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin-gene B33 (Rocha-Sosa et al EMBO J 8. 23-29), which was first of all cloned into the SacI-position of the polylinker of pUC 18. For this the overhanging 3'- end of the Sac-I-cleavage site had been rendered blunt by T4-DNA polymerase. After this the EcoRI-BamHI-fragment was inserted between the EcoRI-BamHI-position of the polylinker of BIN 19.

Fragment B contains the 2909 bp cDNA fragment cBE which codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment from the vector pUC 19 and was cloned into the SmaI-position of the polylinker of BIN 19 after the HindIII-position was filled in with DNA polymerase. For this the orientation of the cDNA was chosen in such a way that the coding strand was readable and a sense-RNA was formed. The cleavage sites BamHI/XbaI and PstI/SphI originate from the polylinker of pUC 19. The cutting positions BamHI/XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI-cleavage sites contained on the fragment B are internal cleavage sites of this fragment.

Fragment C (192 bp) contains the polyadenylation signal of gene 3 of the T-DNA of the Ti-plasmid PtiACH5 (Gielen et al EMBO J 3, 835-846, Nucleotide 11749-11939), which was isolated as Pvu-II-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983) Nature 303, 209-213) and which was cloned between the sphI-HindIII-cleavage site of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII-cleavage site.

The plasmid p33-BE was transferred into Agrobacterium tumefaciens and used for the transformation of potato plants.

Example 4

Preparation of the plasmid p33-anti-BE and introduction of plasmid into the plant genome of potato.

In a similar manner to that described in Example 2, plasmid p33-anti-BE was prepared but replacing the 35S-promoter with the promoter of the class I patatin-gene B33 (Rocha-Sosa et al EMBO J 8, 23-29). The plasmid p33-anti-Be has a size of 14.6 kb and consists of three fragments A, B and C which were cloned into the cleavage sites of the polylinker of BIN 19.

Fragment A contains the DraI-DraI-fragment (position -1512 to position +14) of the promoter region of the patatin-gene B33 (Rocha-Sosa et al EMBO J 8, 23-29) which was firstly cloned into the SacI-position of the polylinker of pUC 18. The overhanging 3'-ends of the SacI-cleavage site were rendered blunt by T4-DNA polymerase. After this the fragment was inserted as EcoRI-BamHI-fragment between the EcoRI-BamHI-position of the polylinker of BIN 19.

Fragment B contains the 2909 bp cDNA fragment cBE which

codes for the branching enzyme. It was cut out as HindIII-SmaI-fragment from the vector pUC 18 and after filling in the HindIII-position with the DNA polymerase, it was cloned into the SmaI-position of the polylinker of BIN 19. For this the orientation of the cDNA was chosen in such a manner that the non-coding strand was readable and anti-sense-RNA could be formed. The cutting positions SphI, PstI and XbaI, BamHI, SmaI originate from the polylinker of pUC 19. The cutting positions BamHI/ XbaI/SalI/PstI originate from the polylinker of BIN 19. The two EcoRI cleavage sites which are located on the fragment B are internal cleavage sites of the fragment.

Fragment C (192 bp) contains the polyadenylation signal of the gene 3 of the T-DNA of the Ti-plasmid pTiACH5 (Gielen et al EMBO J 3, 835-846), Nucleotides 11749-11939), which had been isolated as PvuII-HindIII-fragment from the plasmid pAGV 40 (Herrera-Estrella et al (1983), Nature 303, 209-213) and which was cloned between the SphI-HindIII-cleavage site of the polylinker of BIN 19 after addition of SphI-linkers to the PvuII cleavage sites.

The plasmid p33-anti-BE was introduced in Agrobacterium tumefaciens and was used for the transformation of potato plants.

Example 5

The nucleotides 166-2909 of the 2909 bp cDNA sequence described in Example 1, that codes for the branching enzyme in the HindII-cleavage site of the cloning vector pUC 19 were inserted into the corresponding cleavage sites of the polylinker of the cloning vector pUC 18. This makes possible a fusion of the N-end of the α-peptide of the ß-galactosidase located on the vector with a part of the branching enzyme. The functionality of the resulting

fusion protein was tested in a mutant of Escherichia coli (KV 832) which is deficient in the branching enzyme (Kiel et al Gene 78, 9 - 17). Cells transformed with this construction were plated out on YT-agar plates containing 0.5% glucose. The resulting colonies were stained with Lugolscher solution. The transformed plant cells showed a yellow-red colour in contrast to the blue coloured un-transformed plant cells which indicates the branching activity of the fusion protein (Kiel et al Gene 78, 9-17). An over-production of this protein in Escherichia coli enables the use as technical enzyme.

Claims

1. A plasmid that contains a DNA sequence that contains information that causes changes in the carbohydrate concentration and the carbohydrate composition in regenerated plants, after insertion into the plant genome.

2. A plasmid according to Claim 1 characterised in that the DNA sequence is the coding sequence of a branching enzyme.

3. A plasmid according to Claim 2 characterised in that the branching enzyme is an enzyme having the following sequence:

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                10        20        30        40        50        60
   1  TCAGGAGCGGTCTTGGGATATTTCTTCCACCCCAAAATCAAGAGTTAGAAAAGATGAAAG
  61  GATGAAGCACAGTTCAGCTATTTCCGCTGTTTTGACCGATGACAATTCGACAATGGCACC
 121  CCTAGAGGAAGATGTCAACACTGAAAATATTGGCCTCCTAAATTTGGATCCAACTTTGGA
 181  ACCTTATCTAGATCACTTCAGACACAGAATGAAGAGATATGTGGATCAGAAAATGCTCAT
 241  TGAAAAATATGAGGGACCCCTTGAGGAATTTGCTCAAGGTTATTTAAAATTTGGATTCAA
 301  CAGGGAAGATGGTTGCATAGTCTATCGTGAATGGGCTCCTGCTGCTCAGGAAGCAGAAGT
 361  TATTGGCGATTTCAATGGTAGGAACGGTTCTAACCACATGATGGAGAAGGACCAGTTTGG
 541  AAAGTATGCCACTGCAGACGCCACAAAGTTTGCAGCACCATATGATGGTGTCTACTGGGA
 601  CCCACCACCTTCAGAAAGGTACCACTTCAAATACCCTCGCCCTCCCAAACCCCGAGCCCC
 661  ACGAATCTACGAAGCACATGTCGGCATGAGCAGCTCTGAGCCACGTGTAAATTCGTATCG
 721  TGAGTTTGCAGATGATGTTTTACCTCGGATTAAGGCAAATAACTATAATACTGTCCAGTT
 781  GATGGCCATAATGGAACATTCTTACTATGGATCATTTGGATATCATGTTACAAACTTTTT
 841  TGCTGTGAGCAATAGATATGGAAACCCGGAGGACCTAAAGTATCTGATAGATAAAGCACA
 901  TAGCTTGGGTTTACAGGTTCTGGTGGATGTAGTTCACAGTCATGCAAGCAATAATGTCAC
 961  TGATGGCCTCAATGGCTTTGATATTGGCCAAGGTTCTCAAGAATCCTACTTTCATGCTGG
1021  AGAGCGAGGGTACCATAAGTTGTGGGATAGCAGGCTGTTCAACTATGCCAATTGGGAGGT
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1081 TCTTCGTTTCCTTCTTTCCAACTTGAGGTGGTGGCTAGAAGAGTATAACTTTGACGGATT
1141 TCGATTTGATGGAATAACTTCTATGCTGTATGTTCATCATGGAATCAATATGGGATTTAC
1201 AGGAAACTATAATGAGTATTTCAGCGAGGCTACAGATGTTGATGCTGTGGTCTATTTAAT
1261 GTTGGCCAATAATCTGATTCACAAGATTTTCCCAGACGCAACTGTTATTGCCGAAGATGT
1321 TTCTGGTATGCCGGGCCTTAGCCGGCCTGTTTCTGAGGGAGGAATTGGTTTTGATTACCG
1381 CCTGGCAATGGCAATCCCAGATAAGTGGATAGATTATTTAAAGAATAAGAATGATGAAGA
1441 TTGGTCCATGAAGGAAGTAACATCGAGTTTGACAAATAGGAGATATACAGAGAAGTGTAT
1501 AGCATATGCGGAGAGCCATGATCAGTCTATTGTCGGTGACAAGACCATTGCATTTCTCCT
1561 AATGAACAAAGAGATGTATTCTGGCATGTCTTGCTTGACAGATGCTTCTCCTGTTGTTGA
1621 TGCAGGAATTGCGCTTGACAAGATGATCCATTTTTTTCACAATGGCCTTGGGAGGAGAGG
1681 GGTACCTCAATTTCATGGGTAACGAGTTTGGCCATCCTGAGTGGATTGACTTCCCTAGTG
1741 AGGGCAATAATTGGAGTTATGACAAATGTAGACGCCAGTGGAACCTCGCAGATAGCGAAC
1801 ACTTGAGATACAAGTTTATGAATGCATTTGATAGAGCTATGAATTCGCTCGATGAAAAGT
1861 TCTCATTCCTCGCATCAGGAAAACAGATAGTAAGCAGCATGGATGATGATAATAAGGTTG
1921 TTGTGTTTGAACGTGGTGACCTGGTATTTGTATTCAACTTCCACCCAAATAACACATACG
1981 AAGGGTATAAAGTTGGATGTGACTTGCCAGGGAAGTACAGAGTTGCACTGGACAGTGATG
2041 CTTGGGAATTTGGTGGCCATGGAAGAGCTGGTCATGATGTTGACCATTTCACATCACCAG
2101 AAGGAATACCTGGAGTTCCAGAAACAAATTTCAATGGTCGTCCAAATTCCTTCAAAGTGC
2161 TGTCTCCTGCGCGAACATGTGTGGCTTATTACAGAGTTGATGAACGCATGTCATAAACTG
2221 AAGATTACCAGACAGACATTTGTAGTGAGCTACTACCAACAGCCAATATCGAGGAAAGTG
2281 ACGAGAAACTTAAAGATTCATCATCTACAAATATCAGTACATCATCTACAAAAAATGCTT
2341 ATTACAGAGTTGATGAACGCATGTCAGAAGCTGAAGATTACCAGACAGACATTTGTAGTG
2401 AGCTACTACTACCAACAGCCAATATCGAGGAGAGTGACGAGAAACTTGATGATTCATTAT
2461 CTACAAATATCAGTAACATTGGTCAGACTGTTGTAGTTTCTGTTGAGGAGAGAGACAAGG
2521 AACTTAAAGATTCACCATCTGTAAGCATCATTAGTGATGCTGTTCCAGCTGAATGGGCTG
2581 ATTCGGATGCAAACGTCTGGGGTGAGGACTAGTCAGATGATTGATCGATCCTTCTACGTT
2641 GGTGATCTCGGTCCGTGCATGATGTCTTCAGGGTGGTAGCATTGACTGATTGCATCATAG
2701 TTTTTTTTTTTTTTTTTTAAGTATTTCCTCTATGCATATTATTAGCATCCAATAAATTTAC
2761 TGGTTGTTGTACATAGAAAAAGTGCATTTGCATGTATGTGTTTCTCTGAAATTTTCCCCA
2821 GTTTTGGTGCTTTGCCTTTGGAGCCAAGTCTCTATATGTAATAAGAAAACTAAGAACAAT
2881 CACATATATAAAATGTTAGTAGATTACCA .

4. A plasmid according to any one of the preceding claims, characterised in that the carbohydrates are mono-, di-, oligo- or polysaccharides.

5. A plasmid according to Claim 4 characterised in that the polysaccharide is starch.

6. A plasmid according to Claim 3 characterised in that the branching enzyme alters the amylose/amylopectin ratio of the starch in plant cells and in plants.

7. A plasmid according to Claims 2 or 3 characterised in that the DNA sequence of the branching enzyme is fused to the regulatory sequences of other genes that ensures a transcription of the branching enzyme coding DNA sequence.

8. A plasmid according to Claim 7 characterised in that the DNA sequence of the branching enzyme is fused in inverted direction to the regulatory sequence of other genes thereby the 3'-end of the coding sequence is fused to the 3'-end of the promoter and the 5'-end of the coding sequence is fused to the 5'-end of the termination signal that gives an anti-sense RNA in the plant produced by the branching enzyme.

9. A plasmid according to Claims 7 or 8 characterised in that the regulatory sequences are promoters and termination signals of plant or viral genes.

10. A plasmid according to Claim 9 characterised in that the promoter is a promoter of the 35s RNA of the cauliflower mosaic virus and the termination signal is the 3'-end of the octopine-synthase-gene of the T-DNA of the Ti-plasmid pTiACH5.

11. A plasmid according to Claim 9 characterised in that the promoter is a promoter of the class I patatin-gene B33.

12. Plasmid P35 S-BE (DMS 6143)

13. Plasmid P35 S-anti-BE (DSM 6144)

14. Plasmid P33-Be (DSM 6145)

15. Plasmid P33-anti-Be (DSM 6146)

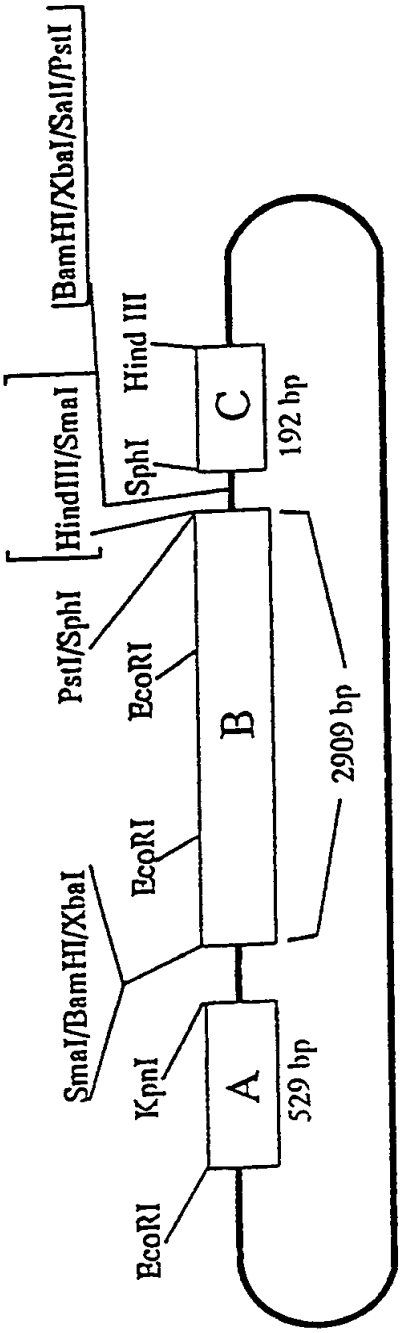
16. A plant that contains a sequence of at least one plasmid according to any one of Claims 1 to 15.

17. A plant according to Claim 16 characterised in that the plants are commercially used plants such as maize, barley, wheat, rice, pea, soya bean, sugar cane, sugar beet, tomato, potato or tobacco.

18. Use of the plasmids claimed in any one of claims 12 to 15, for the production of transgenic plants in which the amylose/amylopectin ratio of the starch is modified.

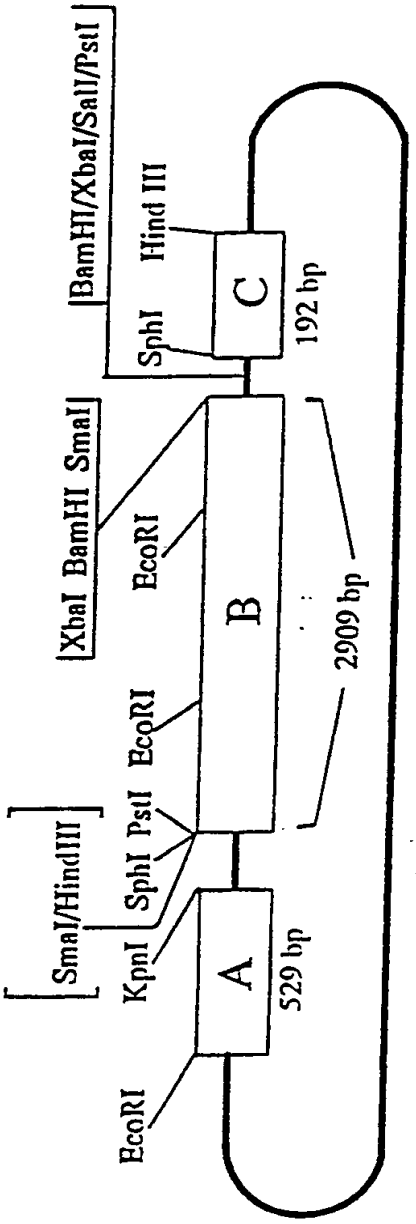
19. Use of the plasmids according to Claim 18 characterised in that the plants are commercially used plants.

20. Use of the plasmids according to Claim 19 characterised in that the plants are maize, barley, wheat, rice, pea, soya bean, sugar cane, sugar beet, tomato, potato and tobacco.



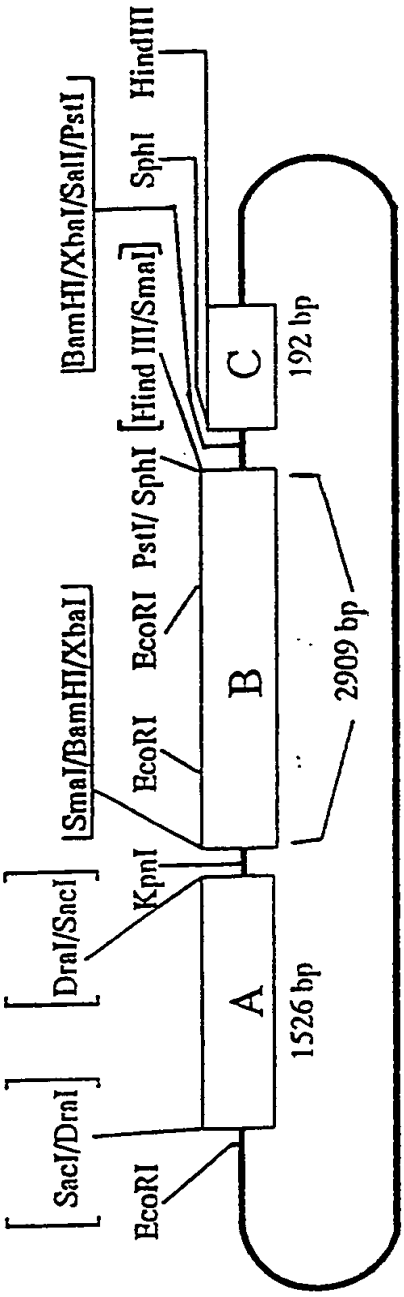
Plasmid p35 S-BE 13,6 kb

Fig. 1



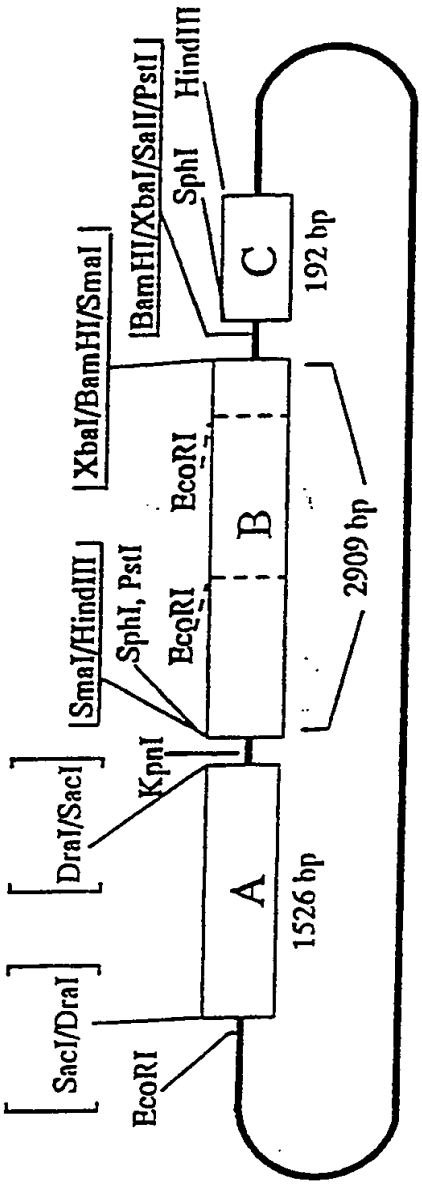
Plasmid p35 S-anti-BE 13,6 kb

Fig. 2



Plasmid p33 - BE 14,6 kb

Fig. 3



Plasmid p33 - anti- BE 14,6 kb

Fig. 4

INTERNATIONAL SEARCH REPORT

International Application No PCT/EP 92/00302

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)*

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 C12N15/82; C12N15/54; C12N9/10; A01H5/00

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	C12N ; A01H

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No.[13]
X	CELL. vol. 60, 12 January 1990, CAMBRIDGE, NA US pages 115 - 122; BHATTACHARYYA, M. K., ET AL.: 'The wrinkled-seed character of pea described by Mendel is caused by a transposon-like insertion in a gene encoding starch-branching enzyme' see the whole document	1,2,4-6
Y		3,7,9, 16,17
Y	WO,A,9 012 084 (DNA PLANT TECHNOLOGY) 18 October 1990 see page 9, line 17	9,16,17

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* Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
15 MAY 1992	27. 05. 92

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	MADDOX A. D.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO [redacted]ELEVANT (CONTINUED FROM THE SECO[redacted]EET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passage	Relevant to Claim No.
Y	PLANT PHYSIOLOGY. vol. 90, 1989, ROCKVILLE, MD, USA. pages 75 - 84; VOS-SCHEPERKEUTER, G. H., ET AL.: 'Immunological comparisin of the starch branching enzymes from potato tubers and maize kernels' see the whole document	3
O,Y	J. CELL. BIOCHEM. SUPPL. vol. 14E, 1990, page 271; VISSER, R.G.F., ET AL.: 'Manipulation of starch in potatoes by new mutants and antisense RNA' see abstract R028	7
X	MOL.GEN.GENET. vol. 225, no. 2, February 1991, pages 289 - 296; VISSER, R. G. F., ET AL.: 'Inhibition of the expression of the gene for granule-bound starch synthase in potato by antisense constructs' see the whole document	1,4,5, 16,17
O,X	THE PLANT CELL. vol. 3, no. 3, 1991, ROCKVILLE, MD, USA. pages 213 - 218; DILWORTH, M. F.: 'Molecular biology comes home' see page 216, right column, last paragraph - page 217, left column & ORAL DISCLOSURE BY L.WILLMITZER, KEYSTONE SYMPOSIUM HELD JAN.10-17, 1991.	1,16,17
X	ABSTRACTS VIITH INTERNATIONAL CONGRESS ON PLANT TISSUE CULTURE AND CELL CULTURE.ABSTRACT A5-36 1990, AMSTERDAM JUNE 24-29, 1990 page 177; VAN DER LEIJ, F.R.,ET AL.: 'Expression of the gene encoding granule bound starch synthase after introduction in an amylose-free and a wildtype potato (Solanum tuberosum)'	1,4,5, 16,17
X	WO,A,9 012 876 (AKTIESELSKABET DANSKE SPRITFABRIKKER) 1 November 1990 see examples 24,25	1,4,5, 16,17
X	WO,A,8 912 386 (CALGENE) 28 December 1989 see the whole document	1,4,16, 17

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III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
P,X	MOL. GEN. GENET. vol. 230, no. 1-2, November 1991, pages 39 - 44; KOSSMANN, J., ET AL.: 'Cloning and expression analysis of a potato cDNA that encodes branching enzyme: evidence for co-expression of starch biosynthetic genes' see the whole document	1-3
P,X	EP,A,0 455 316 (INSTITUT GENBIOLOGISCHE FORSCHUNG BERLIN) 6 November 1991 see the whole document	1,4,5, 16,17

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. EP 9200302 SA 56101

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9012084	18-10-90	US-A-	5034323	23-07-91
		AU-A-	5412390	05-11-90
		EP-A-	0465572	15-01-92
		WO-A-	9011682	18-10-90
WO-A-9012876	01-11-90	AU-A-	5531890	16-11-90
		EP-A-	0470145	12-02-92
WO-A-8912386	28-12-89	AU-A-	3852089	12-01-90
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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82